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(54) Title: EXPRESSION VECTORS CONTAINING THE HIV *nef* GENE TO ENHANCE REPLICATION EFFICIENCY

(57) Abstract

Vectors containing a sufficient number of nucleotides to express HIV gene products necessary to be replication-competent and also containing an ELI *nef* gene segment are disclosed. These vectors can be used to transfect a wide range of cells and hosts for use in assay systems.

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EXPRESSION VECTORS CONTAINING THE HIV *nef* GENE TO ENHANCE REPLICATION EFFICIENCY

The present invention is directed to a vector comprising a replication-competent HIV-1 provirus or HIV-2 provirus and a sequence which enhances the replication efficiency of said provirus.

The human immunodeficiency virus (HIV-I, also referred to as HTLV-III, LAV or HTLV-III/LAV) is the etiological agent of the acquired immune deficiency syndrome (AIDS) and related disorders [Barre-Sinoussi, et al., Science 220:868-871 (1983); Gallo et al, Science 224:500-503 (1984); Levy et al., Science 225;840-842 (1984); Popovic et al., Science 224:497-500 (1984); Sarngadharan et al., Science 224:506-508 (1984); Siegal et al., N. Engl. J. Med. 305:1439-1444 (1981)]. The disease is characterized by a long asymptomatic period followed by progressive degeneration of the immune system and the central nervous system. Studies of the virus indicate that replication is highly regulated, and both latent and lytic infection of the CD4 positive helper subset of T-lymphocytes occur in tissue culture [Zagury et al., Science 231:850-853 (1986)]. The expression of the virus in infected patients also appears to be regulated to enable evasion of the immune response. Molecular studies of the regulation and genomic organization of HIV-I show that it encodes a number of genes [Ratner et al., Nature 313:277-284

(1985); Sanchez-Pescador et al., _Science_ 227:484-492 (1985); Muesing et al., _Nature_ 313:450-457 (1985); Wain-Hobson et al., _Cell_ 40:9-17 (1985)].

Retroviruses are typically classified as belonging to one of three subfamilies, namely oncoviruses, spumaviruses and lentiviruses. Infection by oncoviruses is typically associated with malignant disorders. These viruses typically contain a single-stranded, plus-strand RNA genome of approximately 8,000 to 10,000 nucleotides encompassing the _gag_, _pol_ and _env_, genes, as well as long terminal repeat (LTR) sequences. Oncoviruses typically contain an oncogene. It is generally believed that spumaviruses are not pathogenic _in vivo_, although they induce foamy cytopathic changes in tissue culture. Infection by lentiviruses is generally slow and causes chronic debilitating diseases after a long latency period. Lentiviruses, in addition to the _gag_, _pol_, and _env_ genes possess a number of additional genes with regulatory functions.

The human immunodeficiency viruses (HIV) has been classified as a lentivirus, because it too causes slow infection and has structural properties in common with such viruses. [See Haase, A.T., _Nature_ 322:130-136 (1986)].

HIV-1 shares the _gag_, _pol_ and _env_ genes, respectively with the other retroviruses [Haseltine, W.A., Journal of Acquired Immune Deficiency Syndrome, 1:217-240 (1988)]. HIV-1 also possesses additional genes modulating viral replication. The HIV-1 genome encodes _vif_, _vpr_, _tat_, _rev_, _vpu_ and _nef_ proteins [Haseltine, W.A., Journal of Acquired Immune Deficiency Syndrome, _supra_]. Additionally, the long terminal repeats (LTRs) of HIV contain _cis_-acting sequences

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that are important for integration, transcription and
polyadenylation. Additional cis-acting signals allow
regulation of HIV sequences by some of the novel HIV gene
products [Haseltine, W.A., Journal of Acquired Immune
Deficiency Syndrome, supra; Sodroski et al., Science
231:1549-1553 (1986); Arya et al., Science 229:69-73 (1985);
Sodroski et al., Science 227:171-173 (1985); Sodroski et al.,
Nature 321:412-417 (1986); Feinberg et al., Cell 46:807-817
(1986) Wong-Staal et al, AIDS Res. and Human Retroviruses
3:33-39 (1987); which are all incorporated herein by
reference].

    Nucleotide sequences from HIV-2 and simian
immunodeficiency virus (SIV) also contain the structural
genes, gag, pol, and env as well as regulatory sequences such
as tat, rev, and nef [Guyader, M., et al., Nature,
326:662-669 (1987); Chakrabarti, L., et al., Nature
328:543-547 (1987), which are incorporated herein by
reference].

    The nef gene of the primate immunodeficiency viruses,
HIV-1, HIV-2 and SIV, distinguish these viruses from other
retroviruses. The ungulate lentiviruses, such as the Visna
virus, caprine arthritis, and encephalitis virus (CAEV) and
the equine infectious anemia virus (EIAV), which are the
retroviruses most closely related to these primate
immunodeficiency viruses, lack the region which specifies
nef, namely the long open-reading frame that begins near the
3' end of the envelope (env) and extends into the 3' LTR.

    The nef protein specified by the HIV and SIV viruses
share common features, including post-translational
modification by addition of the fatty acid, myristic acid at
the amino terminus and regions of conserved amino acid

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sequences. The protein encoded by the HIV-1 nef gene is
typically 206 amino acid long, although some variations occur
depending upon the strain. Furthermore, antibodies to nef
protein are found in infected individuals and in infected
monkeys. This would suggest that nef plays an important role
in natural infection. However, this is contradicted by other
data. For example, the nef protein is prematurely truncated
in many replication-competent HIV-1 isolates.

Initial studies using proviruses derived from the IIIB
strain of HIV-1 revealed that viruses that express nef
protein replicate slightly more slowly in CD4$^+$ T cell
cultures than do viruses defective for nef [Terwilliger, E.,
et al. J. Virol. 60:754-760 (1986) which is incorporated
herein by reference]. This finding has been confirmed using
several other pairs of viruses, including an isogenic pair
derived from the BRU and NY5 strains [Luciw, P.A., et al.,
PNAS 84:1434-1438 (1987); Niederman, T.M., et al., PNAS
86:1128-1132 (1989); Ahmad, N., et al., Science 241:1481-1485
(1988); and Cheng-Myer, C., et al., Science 246:1629-1632
(1989), which are all incorporated herein by reference].
Differences of 5 to 10 fold in replication of nef$^+$ as
compared to nef$^-$ viruses have been reported for growth of
these viruses in T-cells. It is also reported that CD4$^+$T
cell lines that constitutively express nef, slow the growth
of many strains of HIV-1 [Cheng, et al., Science 246,
supra]. This difference between nef$^+$ and nef$^-$ viruses,
however, is not always found. In some studies, nef positive
viruses replicated as well or slightly better than nef$^-$
defective viruses.

Although a great deal of research has been expended on
understanding the human immunodeficiency viruses

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(particularly, HIV-1 and HIV-2), there have been problems
with fully understanding their life cycle. Individuals
infected with these viruses typically exhibit a lengthy
asymptomatic period during which viral titers are low and
levels of T4$^+$ lymphocytes are normal, and such a phase
typically extends over a period of years. The virus also
does not cause infection in most animals. While chimpanzees
can be infected, it is very difficult to follow the viruses
in chimpanzees, as they do not readily display signs of
infection and very little viral protein or RNA is made.

Accordingly, it would be extremely useful to have a
vector containing nucleotides corresponding to a
replication-competent HIV provirus and a sequence which makes
it easier to cause infection in a wider range of cells and a
wider range of animals with the provirus in order to be able
to develop animal models for treating the disease.

In particular, it would be useful to use such a vector in
a system where the virus can be followed in vitro and in vivo
in a wide range of cells to screen for drugs that inhibit
infection and to be able to develop drugs that might stop or
minimize infections in certain cells.

It would also be useful to use such a vector in a system
where the virus can be followed in vivo in developing a
viable animal model for studying different aspects of HIV
pathogenesis.

It would also be useful to have a more aggressive virus,
which would provide a more visible target for an animal's
immune system, to provoke a more intense and effective immune
response in order to develop a vaccine and/or antibodies.

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Summary of the Invention

We have now developed a vector comprising a sufficient number of nucleotides corresponding to an HIV genome, other than all structural genes of the ELI strain, to express HIV gene products necessary for viral replication and infectivity (HIV segment) and also containing a sequence of nucleotides corresponding to the nef gene of the ELI strain of HIV (nef gene segment). Preferably, the HIV segment of this vector would also contain a functional vpu gene segment or a functional vpr gene segment. Still more preferably, the HIV segment of this vector would contain both a functional vpu and a functional vpr gene segment.

In a second embodiment we describe a vector comprising an ELI gene segment. Preferably, this vector also contains a heterologous gene.

The vectors can be used in vivo and in vitro to transfect a wide variety of cell lines.

Brief Description of the Drawings

Figure 1 is a schematic diagram of an HIV genome and a series of vectors prepared.

Figure 2 is a schematic diagram of a portion of the HXB ELI 1-fs vectors.

Figure 3 is a schematic diagram of a portion of the HXB ELI 2 and HXB ELI 2-fs vectors.

Figure 4 is a schematic diagram of a vector containing an HIV segment, a functional nef gene, a funcitonal vpu gene, and a functional vpr gene.

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Figure 5 is a schematic diagram of a portion of the HXB-BH8 vector.

Figure 6 is a chart of p24 production in peripheral blood lymphocytes (PBL) over time by a variety of vectors.

Figure 7 is a chart of p24 production in monocyte/macrophage cultures transfected by various vectors over time.

Figure 8 is a chart of p24 production in PBL transfected by various vectors over time.

Detailed Description of the Invention

We have now produced a vector containing a sufficient number of nucleotides corresponding to an HIV genome to express HIV gene products necessary for viral replication and infectivity (referred to as the HIV segment) and a sequence of nucleotides corresponding to a nef gene of the ELI strain to enhance viral replication (referred to as the ELI nef gene segment).

Preferably, the HIV segment corresponds to nucleotides of the HIV-1 or HIV-2 genomes. More preferably, the HIV segment corresponds to nucleotides of the HIV-1 genome. Preferably, the HIV segment does not correspond to the entire ELI strain of HIV-1.

The term corresponding means that conservative additions, deletions and substitutions are permitted.

The nef gene of ELI has its initiation codon located at position 8822, which is immediately after the end of the env

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reading frame and extends through most of the U3 region of
the 3' LTR. This gene encodes a protein with an apparent
molecular weight of about 25-27 kd.

Most studies of nef function have used clones derived
either from the prototype American IIIB strain (Gallo, R.C.,
et al. Science 224:500 (1984); Ratner, L., et al., Nature
313:277 (1985)], the French strain LAV BRU [Barre-Sinoussi,
F., et al., Science 220:868 (1983); Wain-Hobson, S., et al.,
Cell 40:19 (1985)], which shares approximately 98 per cent
sequence homology with IIIB, or the American SF-2 strain
[Levy, J.A., et al., Science 229:840 (1984)];
Sanchez-Pescador, R., et al., Science 227:484 (1985)], which
is over 90 percent homologous with both IIIB and BRU at the
DNA level. All of these are strongly T-cell tropic strains.
In contrast, LAV ELI is one of the more divergent clones in
contrast to the IIIB, SF-2 and BRU strains. The clone is
intact for all the known HIV regulatory functions [Cohen, E.,
et al., Nature 334:532 (1988); Cohen, E.A., et al., J. AIDS,
3:11 (1990), Alizon, M., et al., Cell, 46:83 (1986) which are
incorporated herein by reference]. LAV ELI a French clone
from a Zairian isolate was isolated in 1983 from a 24 year
old woman with AIDS by co-cultivation of patient peripheral
blood lymphocytes with fresh PHA-stimulated PBMC. There are
47 differences in amino acid sequence (out of a total of 207)
between the nef product of LAV ELI and that of the
IIIB-derived Hxorf clone used in our previous studies of nef
[Terwilliger, E.F., et al., J. Virol. 60:754 (1986)].

In preparing a vector according to the present invention
containing a functional nef segment many techniques are
possible. For example, one can use a clone, e.g., LAV ELI,
which contains a functional nef gene. One can obtain this

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segment from the LAV ELI proviral clone by standard
techniques such as by use of restriction endonucleases. For
example, there is a native BamHI site at position 8506 and a
SacI site at position 9607. This segment will contain the
entire nef gene. One can trim this segment so that it
corresponds only to the ELI nef gene segment and not to most
of the other ELI gene segments by standard techniques. For
example, one can insert an Eco RI site position 8792 which is
only 30 base pairs upstream from the nef initiation codon.
One can also use one of the other HIV clones containing nef
sequences more homologous to the ELI strain than the IIIB
strain. As used herein, these nucleotide sequences based
upon nef sequences more homologous to the ELI strain than the
IIIB strain, enhance viral replication and are considered
equivalent to ELI nef. This property can readily be
determined by the skilled artisan based upon the present
disclosure. Alternatively, one can chemically synthesize a
functional "ELI nef segment" nucleotide segment. Other
techniques known to the skilled artisan based on the present
disclosure can also be used to prepare the ELI nef segment.

This ELI nef segment can preferably be inserted in the
presently described vector containing the HIV segment in the
location where the nef segment from the other HIV-1 or HIV-2
strains used would be. If one used an HIV segment with a nef
gene, one can excise that nef gene and insert the ELI nef
segment.

The HIV segment will contain a sufficient number of
nucleotides corresponding to all HIV genes necessary for
replication and infection. Preferably this segment will also
contain nucleotides corresponding to a functional vpu gene or
a functional vpr gene. More preferably, it will contain

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nucleotides corresponding to both functional _vpu_ and _vpr_
genes.

For example, the _vpu_ sequence in the HXB2 provirus does
not contain an initiation codon for _vpu_ and therefore does
not express a functional _vpu_ gene. When the HIV segment has
nucleotides corresponding to HXB2 strain one would insert an
initiation codon immediately upstream and in frame with the
vpr sequence. Alternatively, one could substitute a sequence
of DNA containing a functional _vpu_ gene for a sequence that
does not contain a functional _vpu_ gene. This can be done by
taking advantage of various restriction sites in the
nucleotide sequence. The clones BH8, BH10, LAV ELI, etc.
contain a nucleotide sequence which results in a functional
vpu gene.

The HXB2 strain has a premature termination codon and
does not express a functional _vpr_ protein. If one is using
an HIV segment that corresponds to such a _vpr_ gene, which is
not considered functional, one can insert a functional _vpu_
gene from a strain that expresses a functional _vpr_ gene
product such as LAV BRU, LAV ELI, etc.

As aforesaid, vectors can be synthesized by a variety of
methods known to the person of ordinary skill in the art. A
variety of different vectors which can be prepared are shown
in Figures 1-4.

Different combinations of sequences containing the ELI
nef sequences can be constructed and inserted within the 3'
end of an HIV sequence, such as one having nucleotides
corresponding to the replication competent HXB2 (also
referred to as HXBc2) provirus. Compatible restriction sites

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can be generated by standard techniques where needed such as
by oligonucleotide-directed mutagenesis. For example, a
BamHI site within LAV ELI and a new Eco RI site within both
the LAV ELI and HXB2 sequences. One preferably takes care to
make sure that with each nucleotide change made for
"inserting" a restriction site, the change does not alter the
predicted amino acid sequences encoded by any gene or at a
minimum results in a conservative amino acid change.
Constructs can be confirmed by sequencing. HXB-ELI 1
consists entirely of HXB2 sequences as far 3' as the native
BamHI site at position 8506, within the env and rev gene
sequences, upstream of nef, and LAV ELI sequences between
that position and a Sacl site at position 9607. The
remainder of the 3' LTR sequence is derived from HXB2. See
Figures 1 and 2. In HXB-ELI-2 the 5' junction between the
HXB2 and LAV ELI sequences is shifted downstream to a new Eco
RI site at position 8792, only 30 base pairs (bp) upstream
from the nef initiation codon. See Figures 1 and 3.
HXB-ELI-2 contains only HXB2 sequences as far 3' as this Eco
RI site. The LAV ELI sequence extends from this position 3'
to the Sac 1 site at 9607, as in the case of HXB ELI-1.
These vectors do not contain functional vpr or vpu genes.
However, functional genes can readily be added as discussed
above. For example, excising the sequence in these vectors
corresponding to the sequence between an Apa 1 site at
position 2008 and a Sal 1 site at position 5820 and replacing
it with the corresponding sequence from clone LAV BRU, which
includes a functional vpr reading frame.

     Similarly, the sequence from the vector corresponding to
a sequence between the Sal 1 site 5820 and a kpn 1 site at
position 6382 can be excised and replaced with the
corresponding segment from BH10, which includes a functional
vpu gene. As discussed above, other strategies and

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modifications of this strategy can be used by the skilled
artisan based upon this disclosure.

Proviral plasmids containing the ELI nef gene and the HIV
segment vector described above can readily be made by
inserting this sequence into a plasmid backbone by known
means such as use of restriction enzyme sites.

As used herein the term a sufficient number of
nucleotides permits additions, deletions and substitutions as
long as the claimed functional ability is not lost. For
example, if one is referring to the functional ability of the
nef gene in terms of viral replication, then replication of
the resultant virus must be greater than by an isogenic nef
virus.

The vector is used to transfect a desired cell by
standard techniques. For example, one can transfect a cell
in vitro using, for example, the calcium phosphate
coprecipitation technique or DEAE dextran technique.
Alternatively, this vector can be used to transfect living
cells in vivo. Such techniques are well known to the skilled
artisan.

By use of the nef gene segment of the present invention,
one is able to take an HIV strain, for example, the HXB2
strain, which does not replicate well in peripheral blood
mononuclear cells (PBMC) as well as purified primary human
monocytese or monocytoid cell lines and obtain enhanced
infectivity in such cell lines.

Although not wishing to be bound by theory, it appears
that there is a complex series of interactions between the

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various genes in the HIV genome, which may be responsible for
some of the more acute effects seen with the virus. For
example, one of the strategems proposed by which HIV evades
the immune response in infected individuals is by
establishment of a low-level infectious state in which the
virus either lies dormant or replicates at a very low level
within most infected cells. Accordingly, the virus can
establish a stable infection via integration of the virus
into the host DNA. In order to accomplish such an objective,
the native virus is initially carefully regulated so that it
does not appear too "aggressive". By taking the nef gene
segment of the present invention and inserting it with other
strains or an HIV-2 genome, some of the interactions are
believed to be overcome. Thus, the provirus formed by the
vectors will be more aggressive. For example, we believe
that the IIIB precursor of HXB2 spontaneously mutated to a
nef⁻phenotype. This nef⁻precursor of HXB2 replicated
poorly in PBMC. A compensatory mutation arose in 3' env
sequences of the precursor to HXB2 to produce a virus that
could replicate well in PBMC. The HXB2 virus contains the
original nef mutation as well as the compensatory mutation in
3' env sequences. Furthermore, the 3' env compensatory
mutation has a negative affect on growth in the presence of
closely related nef sequences such as the BH8 and BH10 nef
genes. This negative interaction does not extend to
divergent nef sequences such as the ELI nef gene.

Consequently, sequences in HIV outside of nef affect nef
function. There are differences in nef alleles with respect
to interaction with the extragenic sequences. BH8 and BH10
nef is a negative regulator with HXB2 3' env sequences. BH8
and BH10 nef should be positive regulators with ELI 3' env
sequences. ELI nef is a positive regulator with either HXB2

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or ELI 3' env sequences. ELI nef is an essential gene for
growth in primary monocytes and macrophages. Accordingly,
various tropisms exhibited by HIV strains will not be
exhibited by vectors of the present invention. Thus, such
vectors can be used to transform a wider range of cells than
the strains typically can.

One can use the present vector to generate an antigenic
response in an animal by standard techniques and then use the
antibody generated in treatment.

It has been reported that a cloned SIV_{mac} virus
containing a simple frameshift mutation in nef has a strong
tendency to back-revert in vivo, with restoration of the nef
reading frame. If, as is often proposed, monocytes are a
primary initial target of HIV infection in vivo and/or
infected macrophages form an important long term reservoir
for maintaining a large pool of virus within the system, our
discovey explain why a strong selection pressure for an
intact nef function would exist in vivo, but not necessarily
in vitro, namely the tropism shown by the ELI nef gene.

Although not wishing to be bound by theory, we believe
that major variations in tropism for different types of
CD4-positive cells is in fact a distinct virus-regulated
phenomenon.

Using this vector to transfect cells in vitro or in vivo
can permit an improved system for screening drugs and
understanding the pathogenesis of the virus. Since the range
of the cells transformed by this vector is wider than that
with other laboratory strains such as the IIIB strain, one is
able to explore the affects of a drug on the spread of the

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virus in a wider range of infected cells than is typical.

For example, it can be used in studies in primary cell cultures to determine the mechanism by which the ELI nef allows high-level replication in monocytes. Such information will be of vital relevance to understanding the mechanisms of disease progression in AIDS, particularly in light of evidence suggesting that monocytropic strains of the virus may play a key role in development of the central nervous system disorders often accompanying HIV infection, independent of whether immunological impairment is evident. [Koyanagi, Y., et al., Science 236:819 (1987)].

In one assay one can add a predetermined compound to a cell or bodily fluid and thereafter attempt to transfect those cells with a vector of the present invention. One would add an amount of the vector to the cell that had previously been determined to be effective in transforming such a cell. Thereafter one would measure the level of infectivity of that cell to determine the effectiveness of a compound in preventing the spread of the virus. Alternatively, one can first transfect the cell either in vitro or in vivo with a vector of the present invention thereafter add the predetermined compound to the cells and then determine the effectiveness of that compound in preventing the spread of the virus. The parameters observed include syncytia formation, single-cell killing, levels of supernatant of HIV p24 core protein such as determined by RIA, immunoprecipitation of metabolically labelled viral proteins with AIDS patient serum, etc.

The vector is preferably used in vitro with primary monocytes/macrophages.

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The vector can also be used in vivo to "infect" cells.
In general, the traditional laboratory strains of HIV-1 have
replicated very poorly in traditional animal model systems,
for example, in the SCID-HU mouse system which several
investigators and companies are attempting to develop into a
viable animal model for certain aspects of HIV pathogenesis.
As aforesaid, the present vectors result in proviral clones
which replicate much more aggressively in primary lymphocytes
than the typical lab strains and should replicate much more
effectively in the SCID-HU mice. Other preferred animal
models include mammals other than humans. Preferred examples
of such mammals would include rabbits, mice, rats, cats and
primates. Preferred primates include chimpanzees and
monkeys.

One can readily determine an effective amount of vector
necessary to administer the animal to establish infection.
For example, 0.1 ug to about 10 mg of vector per kg body
weight. Administration can be any of a variety of routes
including parenteral administration such as intramuscular,
intraperitoneal, intravenous or subcutaneous.

Knowing the amount and the conditions necessary to
establish "infection" (transfection) permits the development
of a model assay to establish effectiveness of a preselected
compound in vivo. One would adminster the compound to the
animal by standard means, such as injection. Thereafter, one
would administer a sufficient amount of the vector to
normally establish an infection and observe the animal to
determine whether an infection develops, and if it does, the
degree of infection. Alternatively, one can first administer
a sufficient amount of the vector to establish an infection
in the animal and then administer the preselected compound.

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Thereafter, one would observe the animal to determine the effect of the preselected compound on the "infection" by the vectors.

In another embodiment, one can prepare a vector comprising a sufficient number of nucleotides corresponding to a nef gene of the ELI strain of HIV-1 to enhance viral replication (ELI nef gene segment), a promoter sequence and a polyadenylation sequence, but not containing all the ELI encoding nucleotide sequences. Preferably, the vector contains an LTR sequence, more preferably an HIV LTR sequence. The promoter can be the LTR or a separate promoter such as another viral promoter, e.g., the SL3 promoter. This vector would preferably contain a heterologous gene. In one embodiment, the heterologous gene would be a marker gene. Marker genes are well known in the art, for example, the chloramphenicol acetyltransferase (CAT) gene or a growth hormone such as human growth hormone (hGH). The vector can be cotransfected into a cell with a vector comprising a replication competent HIV provirus, or alternatively into a cell line transfected by a replication competent HIV, e.g., HXB2 or HXB-BH8. This would permit further anlaysis of the interaction between the ELI nef segment and other viral genes. The use of this vector should also increase the range of host that can be transfected by such virus.

The present invention is further illustrated by the following examples. These examples are provided as an aid to understanding the invention, and are not to be construed as a limited thereof.

Examples

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Construction of Vectors

HXB2 is a full-length infectious provirus derived from
the IIIB strain of HIV-1 [Fisher, A.G., et al., Nature
316:262-265 (1985)] its nef, vpr and vpu genes are defective
as a consequence of single point mutations in each gene.
There is a BamHI site at nucleotide 8506, whose location was
used in preparing a variety of plasmids. See Figure 1.

Compatible restriction sites were generated where needed
by oligonucleotide directed mutagenesis - specifically a
BamHI site within LAV ELI and a new Eco RI site within both
the LAV ELI and HXB2 sequences. In each instance changes
were made which did not alter the predicted amino acid
sequence encoded by any gene. All constructs were confirmed
by sequencing.

HXB-ELI 1 consists entirely of HXB2 sequences as far 3'
as the native BamHI site at position 8506, within the env and
rev gene sequences upstream of nef, and LAV ELI sequences
between that position and a SacI site at position 9607. See
Figures 1 and 2. The remainder of the 3' LTR sequence is
derived from HXB2.

In HXB-ELI-2 the 5' junction between the HXB2 and LAV ELI
sequence is shifted downstream to a new Eco RI site at
position 8792, only 30 bp upstream from the nef initiation
codon. See Figures 1 and 3. HXB-ELI-2 contains only HXB2
sequences as far 3' as this Eco RI site. The LAV ELI
sequence extends from this position 3' to the Sacl site at
9607, as in the case of HXB-ELI-1.

The substitution by the ELI sequences also introduced

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changes within the second coding exon of rev [Sodroski, J.G.,
et al. Nature 371:412 (1986)] and that portion of the env
gene encoding the carboxy terminus of the transmembrane
protein, as well as the U3 region of the 3' LTR which
overlaps nef. A frameshift was therefore introduced within
the nef reading frame of the recombinant to generate a second
isogenic clone containing a dysfunctional nef-HXB-ELI 1-nef.
Four base pair frameshifts were introduced within the nef
coding sequences of HXB-ELI-1 and 2 by filing out the native
XhoI site at position 8928 to generate isogenic nef-defective
proviruses. See Figures 2 and 3, respectively.

    In HXB-BH8, the 3' terminus at the native XhoI site
contained sequences derived from the BH8 provirus. This
strain is a IIIB strain, which contains a complete nef open
reading frame and was originally referred to as HXB-orf. See
Figure 5. The vpr$^+$, vpu$^+$ HXB-BH8-R provirus was derived
from the vpr$^-$, vpu$^-$ HXB-BH8. The proviral sequence of
HXB-BH8 between an Apa 1 site at position 2008 and a Sal 1
site at position 5820 was replaced with the corresponding
sequence from clone LAV BRU. This segment includes a
functional vpr reading frame. The HXB-BH8 sequence between
5820 and a kpn 1 site at position 6382 was then excised and
replaced with the corresponding segment from BH10, a closely
related IIIB clone. This segment includes a functional vpu
gene. The same replacements were made in HXB-ELI 1 to
generate HXB-ELI 1-R. See Figure 4.

    The HXB-ELI 1-R sequence was inserted into the known
plasmid pBR322 between the EcoRV and PvuII sites to generate
the plasmid DFCI-HT, which contains the HXB-ELI-1-R sequence
with a pBR322 backbone.

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A sample of this plasmid has been deposited with the
American Type Culture Collection (ATCC) under the Budapest
Treaty and given ATCC Accession No. _68343_____.

Transfection by Vectors

10^7 Jurkat cells were tranfected by the DEAE-dextran
procedure on Day 0 with 10 mg of each proviral plasmid as
described by Lopata, et al., Nucl. Acids Res. 12:5707-5717
(1984); Queen and Baltimore, Cell 33:741-748 (1983);
Sodroski, J., et al., Science 231:1549-1553 (1986). Cultures
were maintained thereafter in 12.5 ml of RPMI +15% fetal calf
serum, and given a complete medium change daily. Aliquots of
each supernatant were saved at the time of each feeding for
later assay. Cultures were monitored for syncytia formation
and total cell number in addition to levels of HIV p24 core
protein the supernatant.

Aliquots of each transfected Jurkat culture were
metabolically labelled, and collected for immunoprecipitation
as described in Terwilliger, et al., J. Virol. 60:754-760
(1986). One-half of each sample was immunoprecipitated with
AIDS patient serum. The other half was reacted with nef
protein antiserum. Immunoprecipitation and polyacrylamide
gel electrophoresis were performed as described by Laemmli,
U.K., Nature 227:680-685 (1970).

Peripheral blood mononuclear cells (PBMC) were isolated
by Ficoll separation from buffy coats obtained from healthy
seronegative blood donors. The PBMC cultures were seeded at
a density of 4×10^6 in 24 mm tissue culture wells (Costar,
MA) in RPMI-1640 with 10% fetal calf serum and antibiotics
and 20% PHA conditioned culture medium. After 48 hours of
culture virus was added to each well. Titered viral stocks

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were obtained from transfection of Jurkat cell cultures.
Infectious supernatant equivalent to 20 ng of p24 was added
to each well of primary cells. The infected cell cultures
were incubated overnight and the growth medium was then
carefully decanted from each well and replaced with fresh
medium. After the last medium change samples for p24 assays
were collected every third day before the cultures were fed.
The samples were inactivated in 0.5% Triton-X and stored
until assayed. p24 was measured using a commercially
available RIΔkit (Dupont, NEN).

Equivalent amounts of cell-free virus were then used to
infect fresh activated peripheral blood mononuclear cells
(PBMC) as well as purified primary human monocytes by the
same means as described above. Replication of each virus was
assayed over time by measurement of p24 core antigen in the
supernatants.

Monocytes were obtained from the PBMC by fractionation on
Percoll gradients. Contaminating T cells were from the low
density cells removed by rosetting with neuraminidase treated
sheep red blood cells.

The purity of the monocyte population was verified by
immunoperoxidase staining of cytopin spin cell preparations
of the isolated monocytes. More than 95% of the cells
stained with Leu3-M and <5% were positive for Leu 18 (CD20),
Leu 3 (CD4), or Leu 2 (CD8). 4×10^6 monocytes were
cultured as described for PBMC cultures to maintain a
comparable growth environment. Viral stocks were used as
described for PBMC cultures and samples for p24 assays were
harvested at the same points.

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The insertion of the 1.1 kb segment of the 3' LAV ELI
sequence in creating HXB-ELI 1, as aforesaid, introduced
changes within the second coding exon of rev and that portion
of the env gene encoding the carboxy terminus of the
transmembrane protein, as well as the U3 region of the 3' LTR
which overlaps nef. The frameshift vector HXB-ELI 1-fs was
generated to result in an isogenic clone containing a
dysfunctional nef. This pair of provirus constructs was then
transfected into Jurkat cells as described above and the
kinetics of virus replication were compared.

The pair of IIIB-derived nef-positive (HXB-BH8) and
negative (HXB2) viruses were also transfected as controls.
As discussed, HXB2 contains a point mutation within nef
resulting in a premature termination codon being inserted
into the reading frame after amino acid 123. This truncated
nef protein is not functional. HXB-BH8 (previously called
Hx-orf) does not contain this mutation, and produces a
full-length nef product [Terwilliger, E.F., et al., J. Virol.
60:754 (1986)].

A lag in levels of supernatant p24 was detectable in the
culture transfected with HXB-BH8 as compared to the one
containing HXB2 at early times post-transfection. A marked
difference in the pattern of syncytia formation was also
apparent. Replication of virus derived from HXB-ELI 1
following transfection was not substantially different from
that of HXB2. In different experiments the kinetics of virus
infection exhibited by HXB-ELI 1 were either equivalent to or
modestly enhanced over those displayed by HXB2.

However, by every parameter measured, the replication of
virus derived from the isogenic nef-defective recombinant

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provirus (HXB-ELI 1-fs) was markedly attenuated. Supernatant
p24 levels in the nef-negative virus culture were depressed
up 100 fold in this experiment compared with the isogenic
nef-positive virus culture. Peak cell-associated viral
protein production was also dramatically decreased in this
virus culture, indicated by the fainter viral bands in the
immunoprecipitation profiles. These measurements paralleled
a dramatic attentuation of the cytophathic effect of the
HXB-ELI-nef⁻ virus upon the infected cells, whether
evaluated by syncytia formation or by decreases in total cell
numbers over time. Similar results were obtained in repeat
experiments, sometimes with even larger differences in
kinetics between the nef-positive and nef-negative HXB-ELI 1
clones. Immunoprecipitation of metabolically labelled
infected cell lysates with a nef-specific polyclonal rabbit
serum revealed the presence of a 27kD nef protein in lysates
from cells infected with either the HXB-BH8 or HXB-ELI 1
viruses. This band was not visible in lysates from cells
transfected with either HXB2 or HXB-ELI 1-fs.

    Transfection by HXB-BH8 confirmed the earlier report that
introduction of a functional nef gene into the HXB2 provirus
results in a virus which replicates more slowly on CD4⁺ T
cells than does the nef⁻ counterpart.

    The LAV ELI nef gene appeared to display a marked
enhancing effect upon the replication of the virus in the
Jurkat cells. This positive effect was larger and in the
opposite direction from the smaller negative effect of IIIB
nef observed in the context of the HXB-BH8/HXB2 pair of
viruses. A repeat experiment using independently derived
clones of the HXB-ELI 1 pair of proviruses yielded comparable
results, eliminating the possibility that a random secondary

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change at some distal site within one of the constructs was
responsible for the results.

   To minimize the potential for generating artifacts in our
experimental design, additional provirus constructs were
prepared as described above. The kinetic studies were also
extended into freshly cultured primary cells challenged with
cell-free virus, as opposed to transfected stable T-cell
lines. HXB-ELI 2 contains a smaller insertion of LAV ELI
sequences than HXB-ELI 1 (See Figure 1 and 3). The 3' end of
the 800 base pair insert remains the same but the 5' end is
shifted downstream to just 30 base pairs 5' to the nef
initiation codon. In addition to the 47 differences in the
predicted amino acid sequences of the nef proteins between
HXB-ELI 2 and HXB2, there is only a single amino acid change
in the predicted env product of HXB-ELI 2. An isoleucine in
the HXB2 env product at position 854, only 2 residues before
the carboxy terminus of gp41, is converted to a serine in
HXB-ELI 2. There are no changes in any other coding frames.
An isogenic nef-defective form of the IIXB-ELI 2 provirus was
also generated by insertion of a frameshift into nef, HXB-ELI
2-fs as described above.

   As aforesaid, HXB2 is defective in two other regulatory
genes in addition to nef, the vpu [Cohen, E.A., et al., J.
AIDS 3:11 (1990)] and vpr [Alizon, M., et al., Cell 46:83
(1986)] genes. In the HXB-BH8-R (for restored) provirus,
which was derived from HXB-BH8, intact vpr and vpu frames
have been restored by recombination with sequences from other
IIIB clones and LAV ELI as described earlier (See Figure 1).
HXB-BH8-R therefore represents a provirus which would be
predicted to be fully functional for all the HIV genes.
Identical recombinations were made in HXB-ELI 1 to generate

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HXB-ELI 1-R. See Figure 4. HXB-BH8-R and HXB-ELI 1-R are
therefore isogenic except for the 1.1 kb of LAV ELI sequence
in HXB-ELI 1-R which replaces the corresponding IIIB sequence
in HXB-BH10-R.

IIIB replicates very well in T-cell lines but poorly in
PBMC, and is particularly weak in either primary monocytes or
monocytoid cell lines [Koyanagi, Y. et al., Science, 236:819
(1987)]. Barely detectable levels of p24 antigen production
were observed within the supernatants of PBMC infected with
the HXB2 virus. See Figure 6. Similarly, the HXB-BH8 and
HXB-BH8-R viruses also did not exhibit significantly
increased activity in the PBMC over HXB2 (Figure 6). No
detectable replication by any of these three viruses was
observed in the monocyte macrophage cultures (Figure 7).

Figures 6 and 7 both show p24 production over time for
cells transfected with HXB-ELI 1 (ELI-1 nef$^+$, open circle),
HXB-ELI 2 (ELI-2 nef$^+$; solid square) HXB-BH8 (BH8 nef$^+$,
open square); HXB-ELI 1-fs (ELI-1fs nef$^-$, solid circle);
HXB-ELI 2-fs (ELI-2fs nef$^-$, solid triangle) and HXB2 (HXBc2
nef$^-$, open triangle).

In contrast, HXB-ELI 1 and HXB-ELI 2 viruses exhibited
strong replication within the PBMC, attaining maximum levels
of p24 antigen production, of ca. 26 ng/ml and ca. 33 ng/ml
respectively, on Day 17 and 20 following infection (Figure
6). In repeat experiments there was no consistent difference
in the level of activity of the two viruses. The HXB-ELI 1-R
virus was even more aggressive, achieving a peak virus
production level 3-5-fold higher than HXB-ELI 1 or ELI 2.
This was more than 50-fold higher than the maximum virus
titer attained by any of the viruses containing IIIB nef

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sequences. In contrast, in cultures infected with either of
the isogenic nef-frameshift HXB-ELI 1-fs and HXB-ELI 2-fs
viruses, supernatant p24 levels were again either barely
within or below the limit of detection.

All three of the nef-positive HXB-ELI recombinant viruses
replicated with great vigor within the primary monocyte
cultures. For example, the HXB-ELI 1 in a typical experiment
reached peak production levels of about 35 ng/ml in the
monocytes on Day 20 following infection. HXB-ELI 1-R again
attained a several-fold higher maximum titer than the other
two viruses. No p24 could be detected in the supernatants of
monocytes infected with either of the HXB-ELI-nef-defective
isogenic viruses. See, for example, Figure 8, where HXB-ELI
1-R is represented by open squares, HXB-ELI by open triangles
and HXB2 by open circles, which shows the results of another
typical experiment.

The results from the Jurkat, PBMC, and monocyte
infections all highlight a dramatic change in the behavior of
the LAV ELI recombinant viruses as compared with those
viruses containing no LAV ELI sequences, and specifically
indicate that this change requires the presence of the intact
LAV ELI nef gene. Viruses such as HXB-BH8 in which the nef
coding sequences were intact but contributed by IIIB did not
exhibit this new phenotype.

This difference is evident even when comparing the
isogenic nef-positive and nef-negative viruses in the Jurkat
cells, where IIIB nef is seen to exert a small negative
effect upon virus replication while LAV ELI nef produces a
larger positive effect. Most striking however, the presence
of the intact LAV ELI nef gene clearly confers upon the

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recombinant viruses a new tropism for monocytic cells, which
is also reflected in their capacity for vigorous replication
in the PBMC. The level of virus production in infected
monocytes by the HXB-ELI 1-R virus is as high as any reported
for the supposedly monocytropic HIV strains [Koyanagi, Y., et
al., Science, 236:819 (1987), Schwartz, S., et al., PNAS,
86:7200 (1989), Collman, R., et al., J. Exp. Med. 170:571
(1989)].

Although possible that additional overlapping elements
for example, within the U3 region of the 3' LTR may
contribute to the changed phenotype of the LAV ELI
recombinants, because HXB-ELI 2 appears to replicate as well
in the monocytes as HXB-ELI 1, the additional sequence
differences between HXB2 and HXB-ELI 1 present in the env and
rev genes do not appear to contribute to the phenomenon. It
is clear, however, from the negative results obtained with
both of the nef-defective LAV ELI viruses that the intact LAV
ELI nef is essential for the phenotype. In contrast, while
there was an approximately 5-fold enhancement in the level of
virus production in the primary cells by the vpu-positive,
vpr-positive HXB-ELI 1-R virus compared to the parental HXB
ELI 1, which is defective for both these functions,
restoration of vpu and vpr in HXB-BH8 did nothing either to
alleviate the block to replication by this virus in the
monocytes nor to boost the low level of infection in the
PBMC. As previously reported, each of these factors
functions to enhance virus replication [Cohen, E.A., et al.,
J. AIDS, 3:11 (1990), Alizon, M., et al., Cell, 46:83
(1986)]. While coding changes in other reading frames have
also been introduced into HXB-ELI 1-R as compared to the
original HXB-ELI 1, the magnitude of the effects we have
characterized for the vpu and vpr during virus infection of

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Jurkat cells is entirely sufficient to account for the boost in virus titers seen in both the PBMC and monocyte cultures infected with HXB-ELI 1-R.

We believe that major variations in tropism for different types of CD4-positive cells in in fact a distinct naturally occurring, virus-regulated phenomenon.

We successfully duplicated the observation of other investigators that nef can modestly down regulate expression from the HIV LTR in trans. This was accomplished by co-transfection into COS-7 cells of an HIV LTR-CAT construct together with either a nef expression vector or an isogenic vector containing a frameshift in the nef seqeunce. Each plasmid included an SV40 origin of replication. However, similar results were obtained using HIV LTR elements cloned from either HXB2 or LAV ELI, and regardless of which strain of nef was being expressed. Together with the replication studies, this suggests that the down regulatory effects may be secondary phenomena.

It is evident that those skilled in the art, given the benefit of the foregoing disclosure, may make numerous modifications thereof and departures from the specific embodiments described herein, without departing from the inventive concepts and the present invention is to be limited solely by the scope and spirit of the appended claims.

We claim:

1. A vector comprising:

    (a) a sufficient number of nucleotides corresponding to
an HIV genome to express HIV gene products necessary for
viral replication and infectivity, the HIV segment, but not
corresponding to all the genes necessary for viral
replication and infectivity from the ELI strain; and

    (b) a sufficient number of nucleotides corresponding to
a nef gene of the ELI strain of HIV-1 to enhance viral
replication, the ELI nef gene segment.

2. The vector of claim 1, wherein the HIV segment contains a
sufficient number of nucleotides corresponding to a
functional HIV vpu gene to produce functional vpu protein.

3. The vector of claim 1, wherein the HIV segment contains a
sufficient number of nucleotides corresponding to a
functional vpr gene to produce functional vpr protein.

4. The vector of claim 3, wherein the HIV segment contains a
sufficient number of nucleotides corresponding to a
functional vpu gene to produce functional vpu protein.

5. The vector of claim 1, wherein the HIV segment
corresponds to a sufficient number of nucleotides to produce
functional env protein, but wherein it does not correspond to
the env nucleotides of the ELI strain.

6. The vector of claim 1, wherein the HIV segment
corresponds to a sufficient number of nucleotides to produce
functional gag protein, but wherein it does not correspond to
the gag nucleotides of the ELI strain.

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7. The vector of claim 1, wherein the HIV segment corresponds to a sufficient number of nucleotides to produce functional pol protein, but wherein it does not correspond to the pol nucleotides of the ELI strain.

8. The vector of claim 1, wherein the HIV genome is the HIV-2 genome.

9. The vector of claim 1, wherein the HIV segment corresponds to nucleotides of the HIV-1 genome.

10. The vector of claim 1, wherein the HIV segment contains a sufficient number of nucleotides corresponding to the gag gene, the pol gene, the env gene, the rev gene, the tat gene, the vpu gene, and the vpr gene to produce functional gag, pol, env, rev, tat, vpu and vpr proteins.

11. A vector comprising:
    (a)   a sufficient number of nucleotides corresponding to a nef gene of the ELI strain of HIV-1 to enhance viral replication, the ELI nef gene segment;
    (b)   a promoter sequence; and
    (c)   a polyadenylation sequence;
wherein the vector does not contain all the ELI encoding nucleotide sequences.

12. The vector of claim 11, wherein the vector contains a sufficient number of nucleotides corresponding to an LTR sequence.

13. The vector of claim 11, wherein the vector also contains a heterologous gene.

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14. A method of assaying in vivo for compounds effective against infection by HIV virus, which comprises:

    (a)   administering to an animal the vector of claim 1, in a sufficient amount to infect that animal;

    (b)   adding a predetermined compound to the animal, and

    (c)   observing the animal to determine the effect of the predetermined compound on infection by the vector.

15. A method of assaying for the effectiveness of compounds against infection by HIV virus, which comprises;

    (a)   adding a predetermined compound to a preselected animal;

    (b)   administering to the preselected animal the vector of claim 1 in an amount which has been determined as sufficient to cause infection, and

    (c)   observing the animal to determine the effect of the preselected compound on infection by the vector.

16. The method of claim 14, wherein the animal is a mammal, other than a human.

17. The method of claim 16, wherein the mammal is a primate.

18. The method of claim 16, wherein the mammal is selected from the group consisting of rabbits, mice, rats, cats, chimpanzees, and monkeys.

19. The method of claim 14, wherein the animal is a chimpanzee.

20. The method of claim 15, wherein the preselected animal is a mammal, other than a human.

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21. The method of claim 15, wherein the mammal is a primate.

22. The method of claim 15, wherein the mammal is selected
from the group consisting of rabbits, mice, rats, cats,
chimpanzees, and monkeys.

23. The method of claim 15, wherein the preselected animal is
a chimpanzee.

24. A method of assaying for compounds effective against HIV
virus, which comprises;
    (a)   transfecting a cell line with a sufficient quantity
of the vector of claim 1 to obtain infected cultures;
    (b)   adding a predetermined compound to the infected
cultures; and
    (c)   screening the cultures to determine the effect of
the compound on the transfected cultures.

25. The method of claim 24, wherein the cell line is a
primary monocyte.

26. A method of assaying for the effectiveness of a compound
against the infectivity of HIV virus, which comprises;
    (a)   adding a predetermined compound to a culture of
cells;
    (b)   attempting to transfect the culture of cells of
step (a) with the vector of claim 1 in an amount, which has
previously been determined to be sufficient for transfecting
the culture of cells; and
    (c)   screening the culture to determine whether the
culture has been transfected and, if it has been transfected,
the extent of the transfection by the vector of step (b).

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27. The method of claim 26, wherein the culture of cells is a
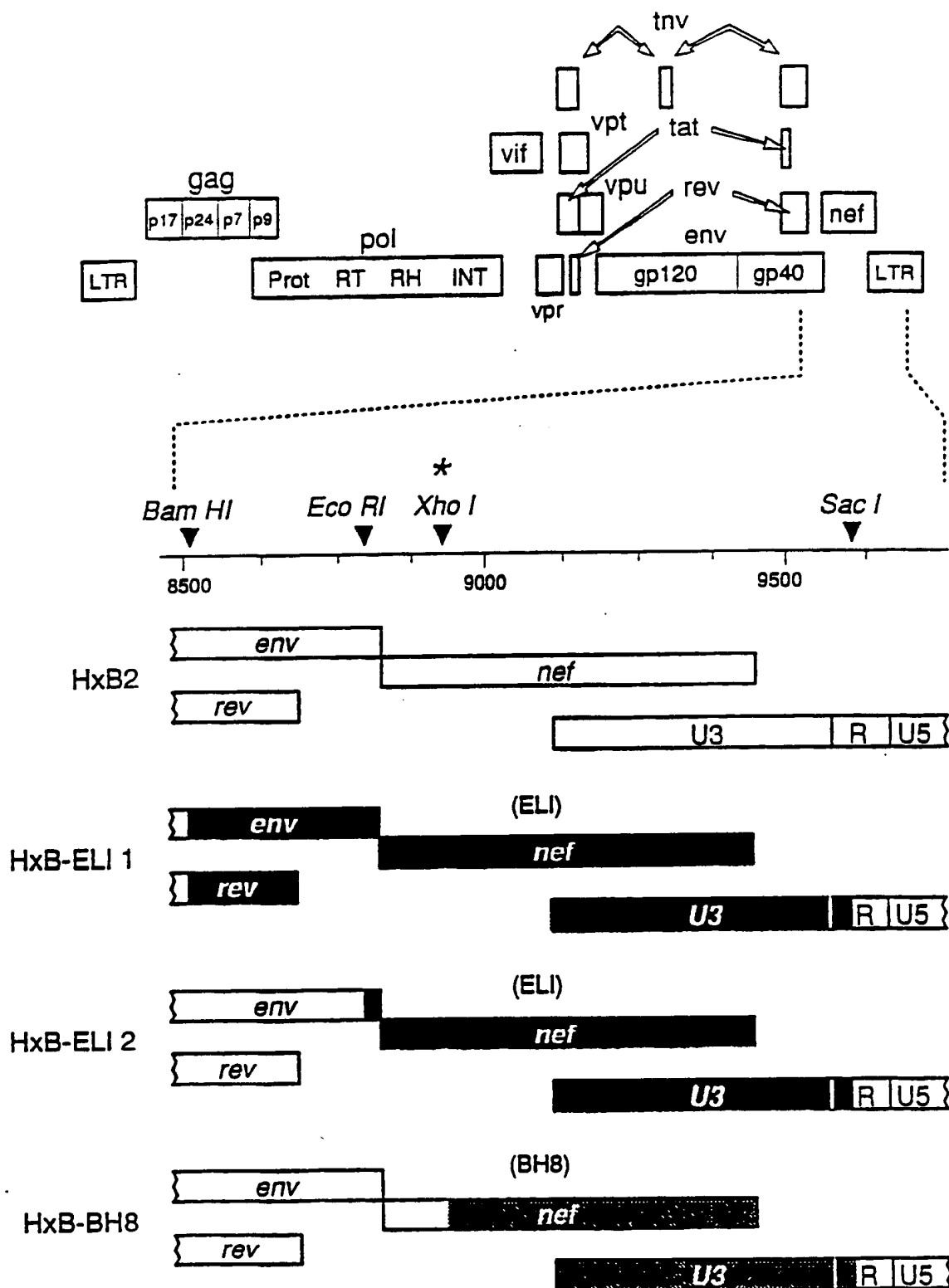culture of primary monocytes.
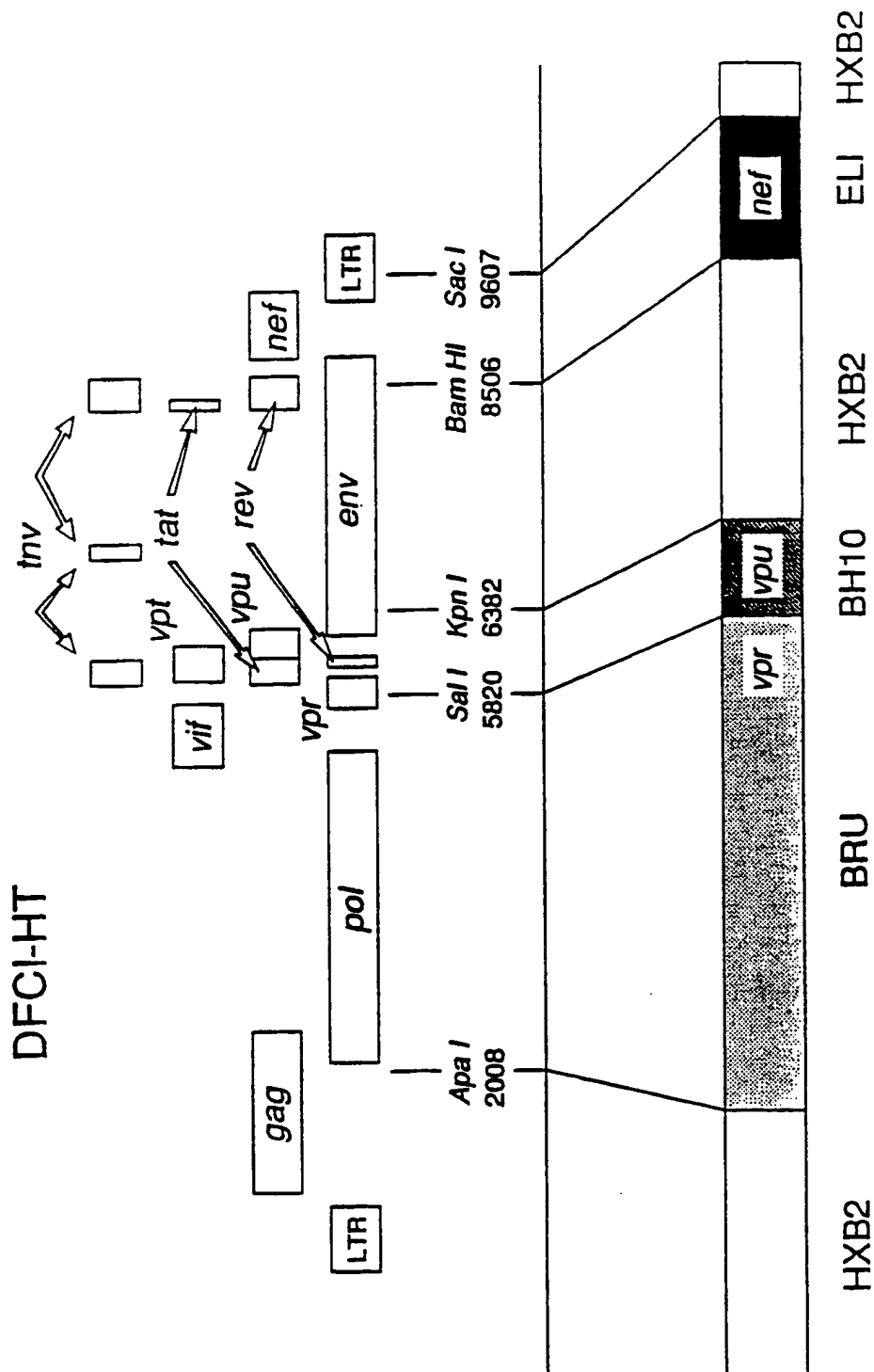
FIGURE 1
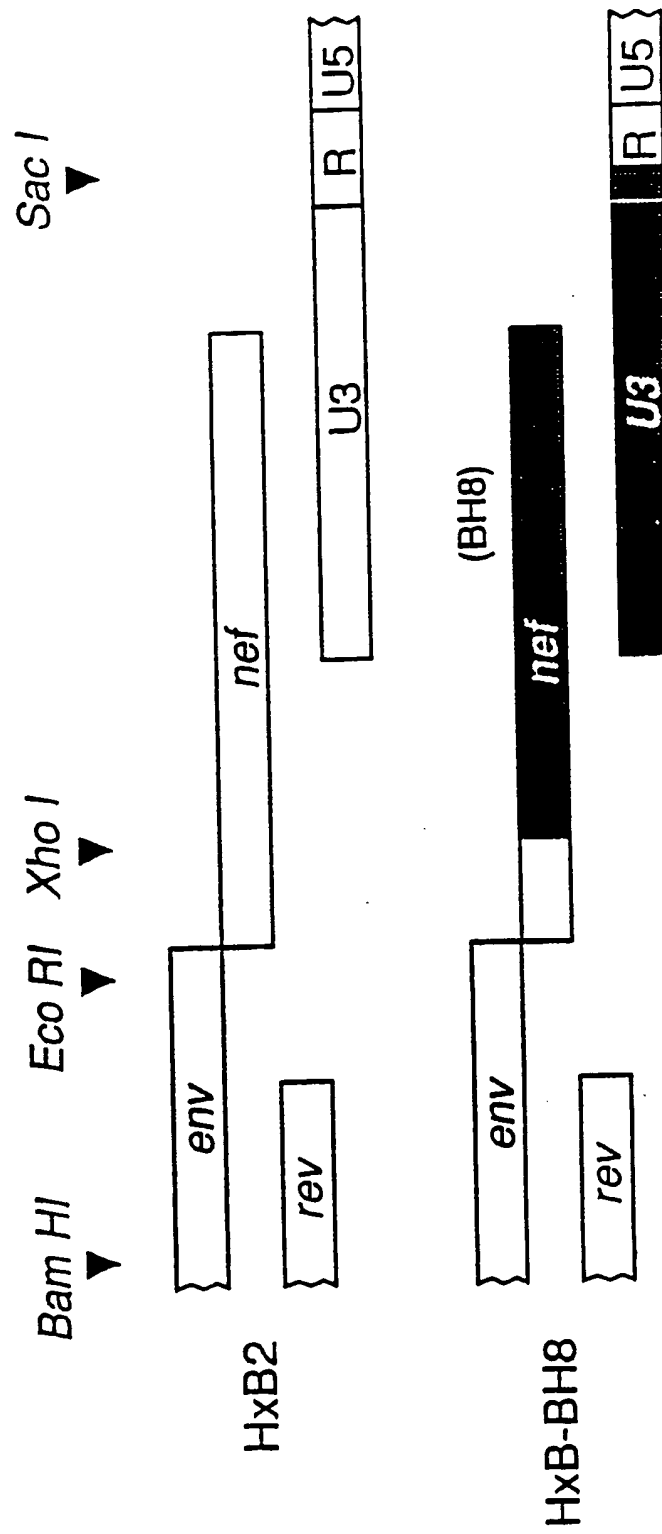
FIGURE 2

FIGURE 3
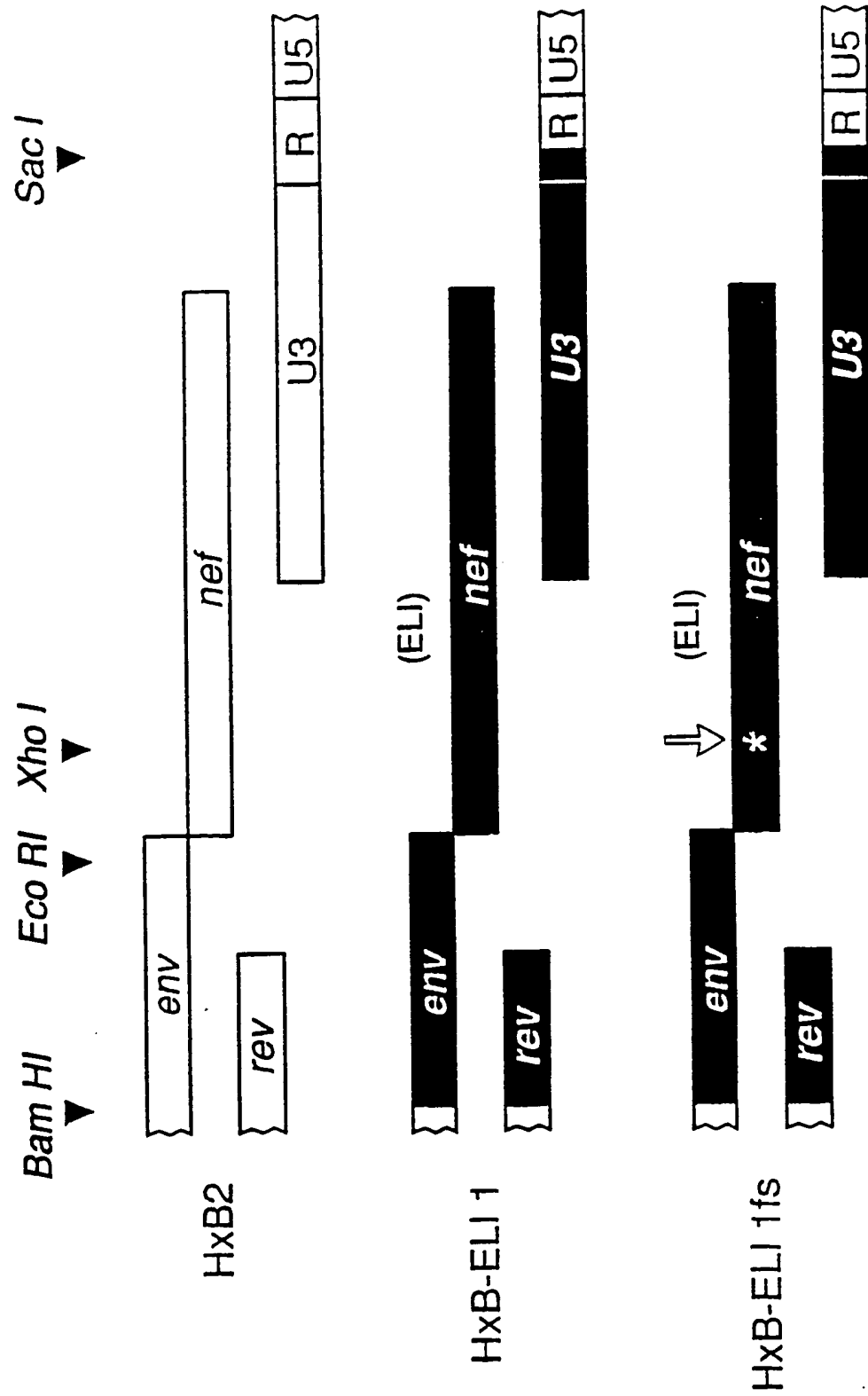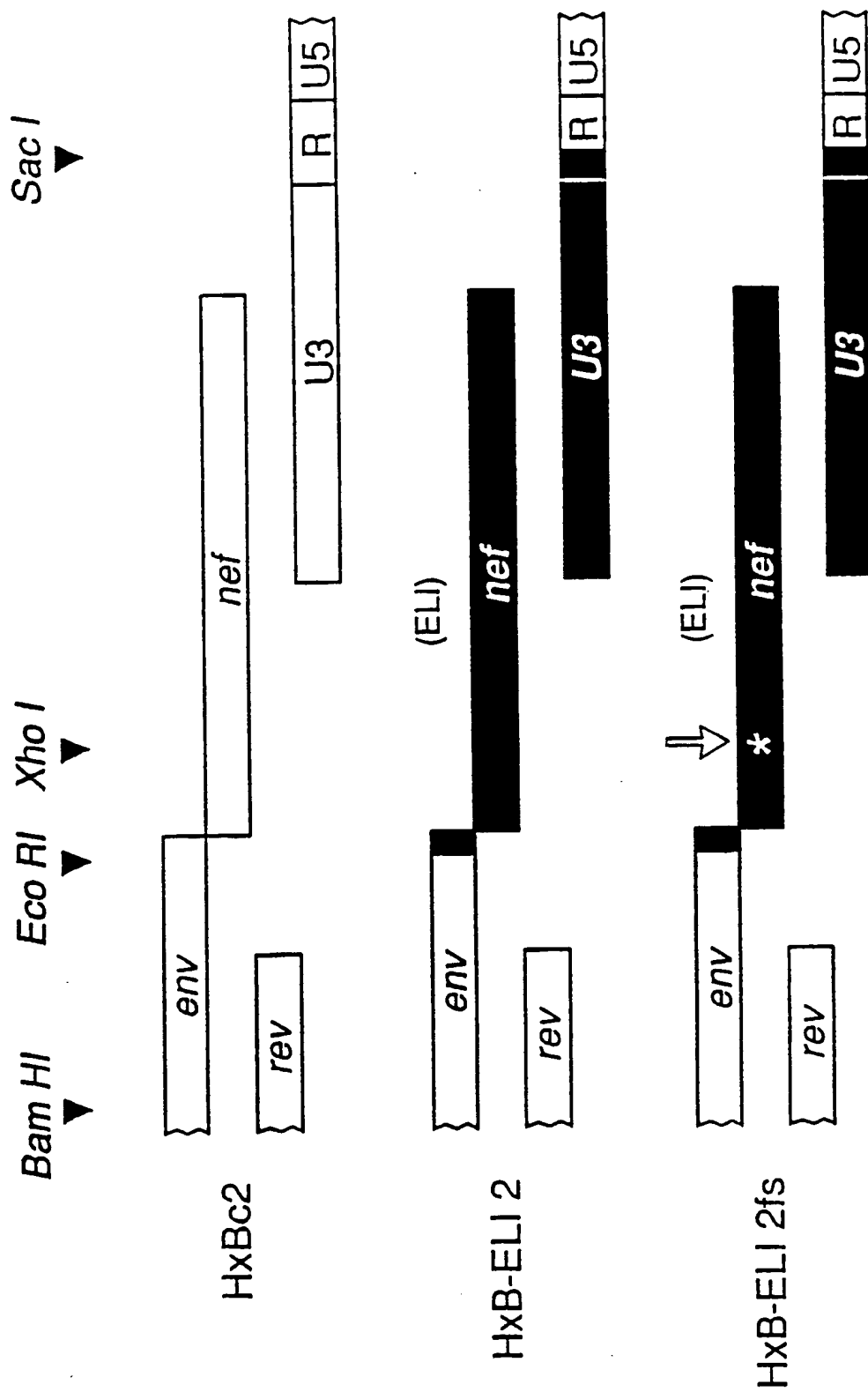
FIGURE 4

FIGURE 5
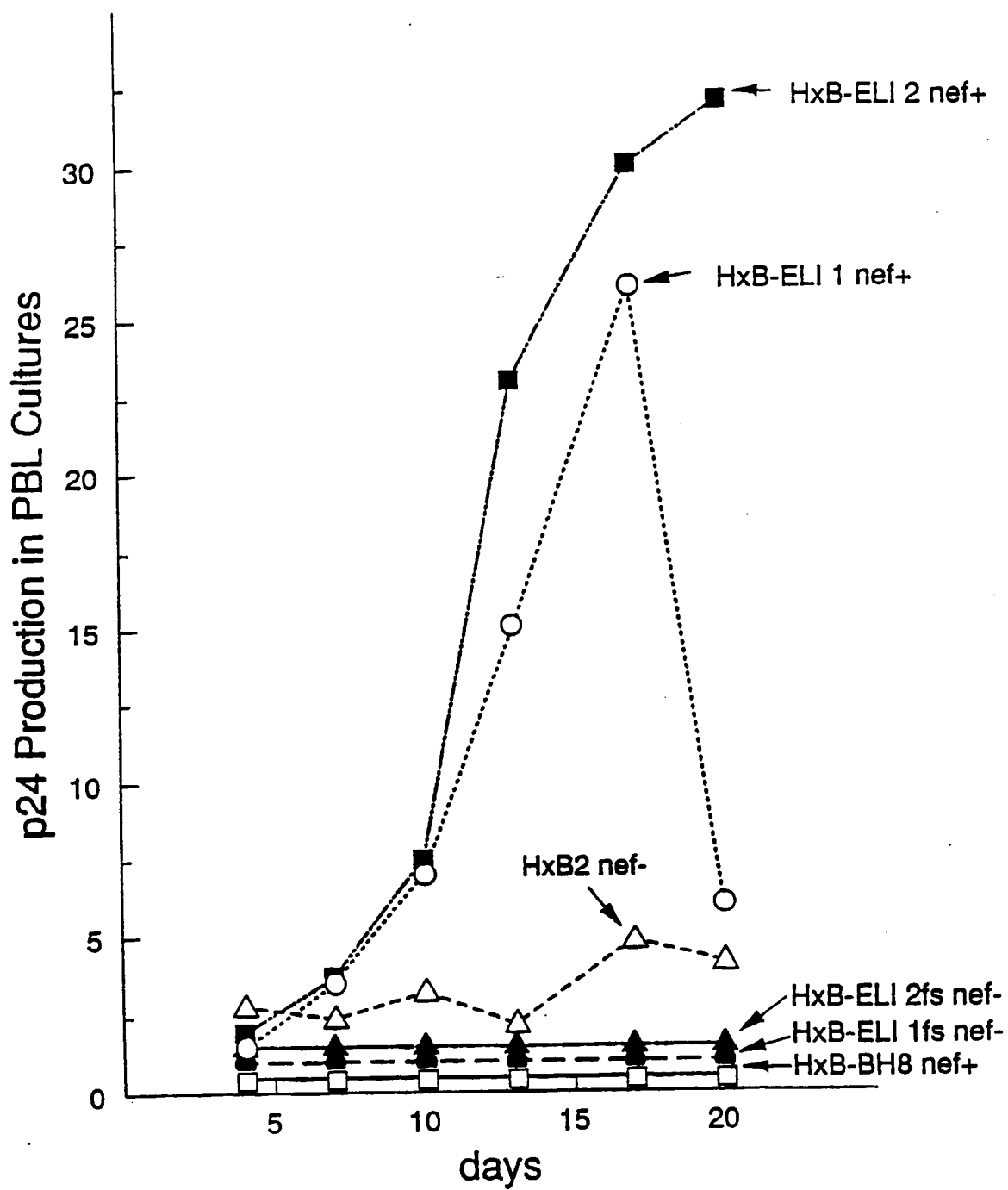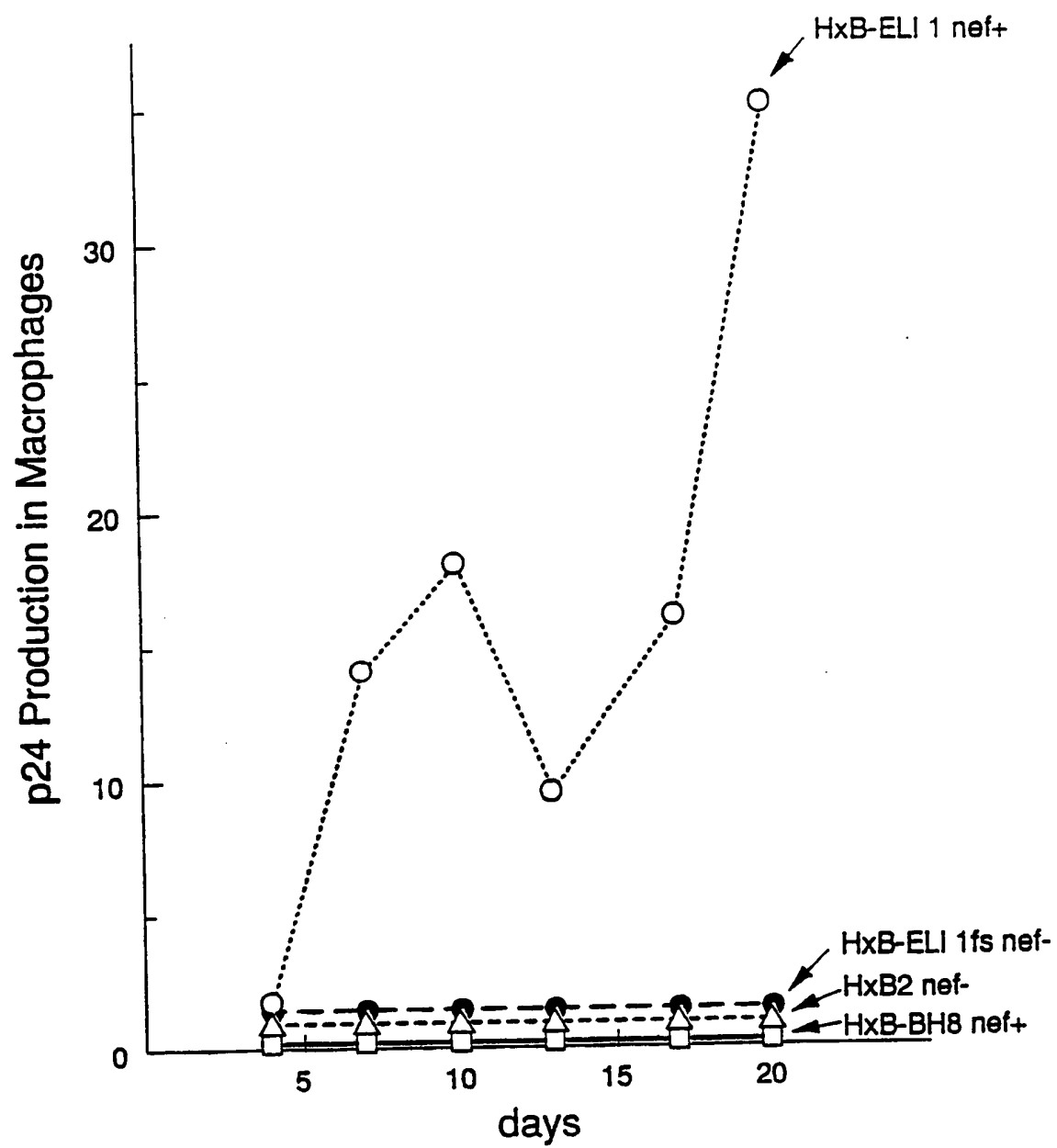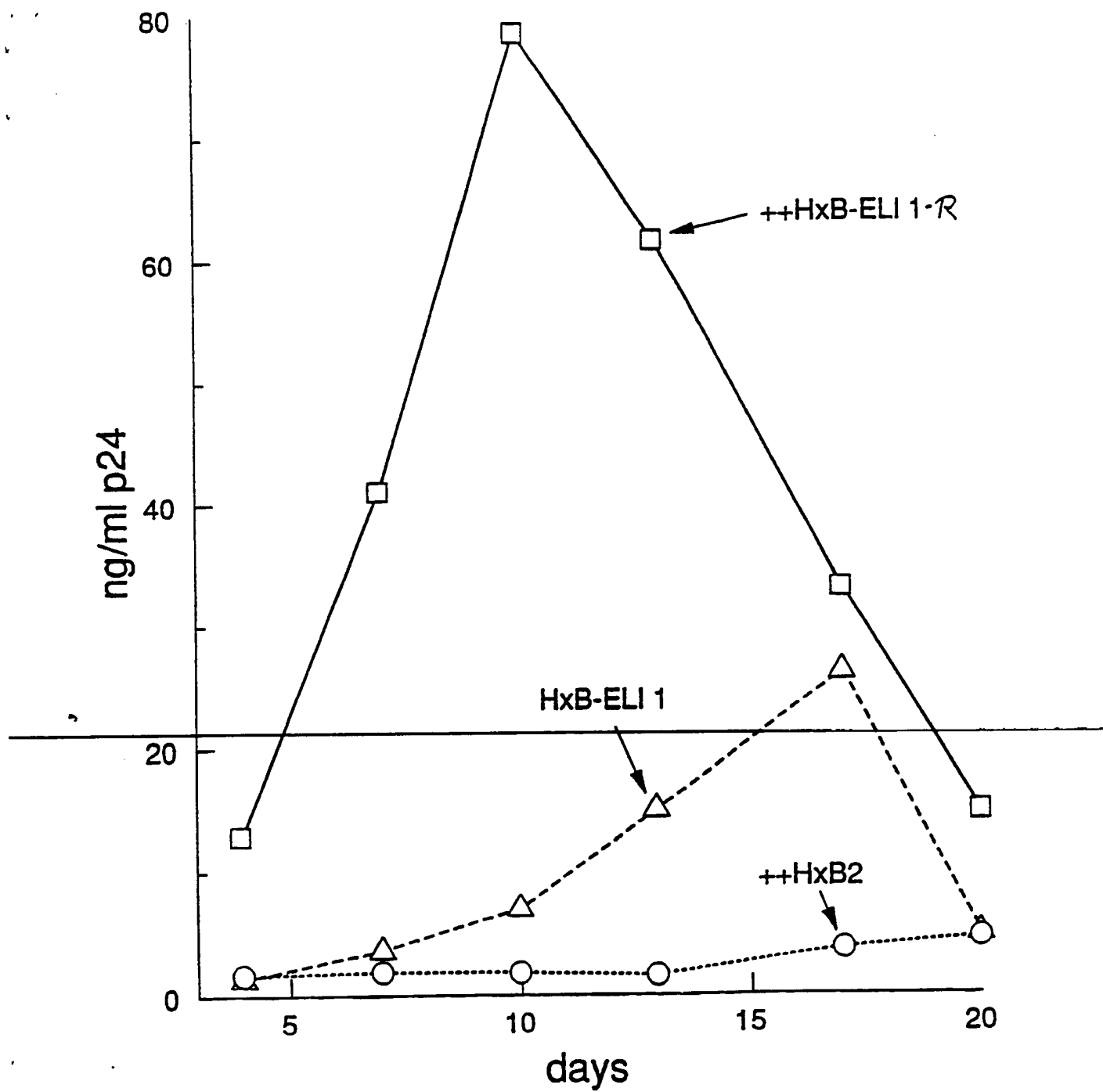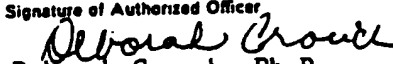
FIGURE 6

FIGURE 7

FIGURE 8

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/04333

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5):   C12Q 1/68;   C12N 15/00
U.S.Cl: 435/6, 172.3, 320.1, 240.2

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.Cl	435/6, 172.3, 320.1, 240.2

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [8]

U.S. Patents and Chemical Abstracts

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Proceedings National Academy of Science, Vol. 86, issued February 1989, Niederman et al. "Human Immunodeficiency Virus Type 1 Negative Factor is a Transcriptional Silencer," pages 1128-1132, see entire document.	1-13
X	Science, Vol. 246, issued 22 December 1989, Cheng-Mayer etal. "Differential Effects of nef of HIV Replication: Implications for Viral Parthenogenesis in the Host," pages 1629-1632. See entire document.	1-13

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish-the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

29 October 1991

Date of Mailing of this International Search Report

25 NOV 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

Deborah Crouch, Ph.D.

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	Nature, Vol. 316, issued 18 July 1985, A. Fisher et al., "A Molecular Clone HTLV III with Biological Activity," pages 262-265, see entire document.	1-13
X	Science, Vol. 247, issued 02 February 1990, J.M. McCune et al., Suppression of HIV Infection in AZT- Treated Scidhu Mice", pages 564-566, see entire document.	14-23

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers   , because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers ...., because they relate to parts of the international application that do not comply with the prescribed require-ments to such an extent that no meaningful international search can be carried out ¹³, specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	US. A, 4,795,739 (Lifson et al) 03 January 1989, especially col. 3, 4, 15, 22-24 and the claims.	24-27